CcdAB$_{Sm}$, a Chromosomal Toxin-Antitoxin Module, Mediates Cell Death in _Serratia marcescens_ Multicellular Behavior

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Abstract

Toxin-antitoxin (TA) modules are gene pairs specifying for a toxin and its cognate antitoxin and are found on the chromosomes of many bacteria including pathogens. It has been demonstrated that chromosomal TA systems influence bacterial multicellular behaviors such as biofilm formation and fruiting body formation. However, the role of TA systems in swarming behavior, another type of bacterial multicellular behavior, remains elusive. Swarming is a complex multicellular behavior requires the integration of chemical and physical signals, which leads to the physiological and morphological differentiation of the bacteria from non-motile vegetative cells into highly-motile swarmer cells. Here we demonstrated that the cell viability was dramatically decreased to c.a. 10% in vegetative cell population during swarming development in *Serratia marcescens*. A pair of chromosomal TA module named *ccdAB*<sub>Sm</sub> was identified. Deletion of *ccdAB*<sub>Sm</sub> decreased cell death during swarming and showed precocious swarming phenotype. Interestingly, deletion of *rssBA*, a two-component system regulating swarming initiation, also showed reduced cell death during swarming. Furthermore, the result from RT-PCR provided evidence that transcription of *ccdAB*<sub>Sm</sub> and their upstream genes are negatively regulated via RssAB signaling. Briefly, our preliminary results demonstrated that RssAB signaling may temporally control *S. marcescens* swarming through down-regulation of *ccdAB*<sub>Sm</sub> to activate CcdB<sub>Sm</sub>, leading to programmed cell death in swarming development. Taken together, coupled with our future works, we will provide evidences that RssAB affects cell death through a chromosomal TA module, CcdAB<sub>Sm</sub>, in swarming development.
Chapter 1: Introduction

1.7 Serratia marcescens

*Serratia* species are Gram-negative, rod-shaped bacteria in the family Enterobacteriaceae. *Serratia marcescens* is the primary pathogenic species of *Serratia*. Over the last 40 years, *Serratia marcescens* has become an important cause of nosocomial infection, particularly catheter-associated bacteremia, urinary tract infections and wound infections [1]. *S. marcescens* is noted for the production of a red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) that is now receiving renewed interest as a consequence of its potency as a therapeutic agent [2, 3]. In recent years, the treatment of *S. marcescens* is become more and more complicated by the resistance to multiple antibiotics [4-7]. As the incidence of *Serratia* infections increases, drug-resistance problems may become increasingly important for public health. *S. marcescens* is now considered as an emerging harmful human pathogen [8, 9].

1.8 Bacterial multicellular behavior

In the late 19th century, Harvard microbiologist Roland Thaxter had found the special life cycle might be considered multicellularity exists in particular bacteria such as *Myxococcus* [10]. Followed that, pattern and organized cellular differentiation in the colonies of many bacterial species, including *E. coli*, were also been reported [11]. Bacteria are now known as, while are unicellular organisms, able to communicate with each other and therefore coordinately regulate behavior that results in differentiation at the cellular and multicellular level [12].

Bacteria often thrive in surface-associated multicellular communities that have advantages over individual cells. Among these, biofilms are sessile communities with microorganisms embedded within a matrix and attached to a surface. However, motile populations, such as swarming bacteria, can rapidly reach novel niches, which they
can colonize; this provides ecological advantages to the bacteria [13, 14].

*S. marcescen* utilizes different multicellular behavior such as swarming motility and biofilm formation to adapt to diverse environments [13, 15]. To study the underlying mechanism by which bacterial movement over surfaces and the motivation of such a complicated behavior in *S. marcescens* is the main stream of this laboratory.

1.9 Bacterial programed cell death

The phenomenon of bacterial cell death and lysis has been the subject of study for well over 100 years [21]. It has been proposed that autolysis fulfills a function in bacterial physiology that is similar to the function of programmed cell death (PCD) in more developmentally complex eukaryotes [22]. The genetically programmed elimination of eukaryotic cells has been shown to be an essential part of development and has an important role in the elimination of damaged cells, which is necessary so that these cells do not become a burden to the population as a whole [23]. However, why would bacteria exhibit such altruistic behavior? The answer to the question could lie in the multicellularity of bacteria. It has been suggested that in the multicellular structures such as biofilm community could provide the selective pressure that is required to maintain PCD by eliminating damaged individuals from the population and thus enhancing the availability of nutrients for the healthy individuals that remain [24].

1.10 Toxin-Antitoxin systems

The term “toxin-antitoxin (TA) module” is used to describe a family of gene pairs whereby one gene encodes an unstable antitoxin that inhibits the potentially lethal action of its cognate toxin encoded by the second gene. These genes were originally identified on extrachromosomal DNA such as low-copy number plasmids and prophages and function to ensure the propagation of these otherwise nonessential genetic elements by killing cells that no longer harbor them [25]. These TA genes
have also been dubbed “addiction modules” since their lethal action causes the bacterial host to be “addicted” to the ongoing presence of these extrachromosomal elements [26, 27].

More recently, several common families of TA modules have been identified on the chromosomes of bacteria and archaea: relBE, higBA, mazEF, ccdAB, vapBC, parDE, phd–doc and yoeB–yefM. Genetic and structural analyses have shown evolutionary relationships between several TA families [28-31]. TA modules in bacteria can be induced by stress conditions such as nutrient stress and antibiotics and act as a mediator of programmed cell death [32, 33]. The function of chromosome-borne TA systems has been highly debated but a central role in bacterial stress physiology is emerging. Their importance in bacterial stress physiology is now widely accepted and their molecular modes of actions are being elucidated [25].

Among the all known TA systems, CcdAB is the best known and has been applied in molecular cloning techniques [34, 35]. CcdB has been found to function as a gyrase poison, turning this crucial enzyme into a harmful agent [36]. The toxin acts as a wedge that stabilizes a dead-end covalent gyrase–DNA adduct [37]. This complex forms an obstruction for DNA and RNA polymerases [38]. The antidote CcdA in the ccd system competes with gyrase for binding the toxin CcdB. CcdB stalls the activity of gyrase by stabilizing a covalent gyrase–DNA adduct that forms a ‘roadblock’ for DNA replication and transcription. Retraction of CcdB from gyrase by CcdA, a process called ‘rejuvenation’ [39, 40], is sufficient to resolve these covalent adducts and to restore the normal activity of gyrase.

1.11 Two-component system RssA-RssB regulates swarming behavior in S. marcescen

Two-component systems are one of the most predominant means by which bacteria sense, respond and adapt to environmental changes [16, 17]. Typically,
two-component systems consist of a sensor protein (histidine kinase) that transfers a high-energy phosphoryl group to the response regulator, which is often a transcription factor [17].

Bacterial swarming is a cell-density dependent multicellular surface migration behavior comprising at least a swarming lag and actively swarming phases [18, 19]. Through the approach of transposon mutagenesis, a pair of two-component signal transduction proteins, RssA-RssB was identified. When mutated, bacteria swarm earlier than the parental strain CH-1, exhibit precocious swarming behavior, suggested that RssA-RssB controls the duration of the swarming lag period [19, 20]. 

\( rssA \) specifies RssA, a histidine kinase, while \( rssB \) specifies the cognate response regulator, RssB [20]. Upon activation, RssA autophosphorylates at His248 followed by transfer of the phosphoryl group to RssB at Asp51 [20]. Furthermore, the phosphoryl transfer is essential for RssA-RssB in regulating swarming behavior [20]. Activation of RssA-RssB signaling prohibited swarming and once signaling was inactivated, surface migration was initiated (Tsai et al., unpublished data).

1.12 Hypothesis and experimental design

It has been suggested that in the development of bacterial multicellularity during swarming lag period, the cells are nonmotile called vegetative cells [41-43]. Moreover, as the bacteria population expands, a second edged swarmer cell population emerges that is highly motile, and physiologically and morphologically distinct from the non-motile, internal vegetative population [18, 44, 45]. Since these vegetative cells are nonmotile, we questioned if the cells are viable or most of them are not. A LIVE/DEAD stain technique (Invitrogen) was applied to assess cell viability in the development of swarming behavior. Surprisingly, we observed numerous dead cells in the population of vegetative cells compared to swarmer cells or cells in overnight LB broth culture. Interestingly, deletion of a putative chromosomal TA module, \( ccdAB_{Sm} \), resulted in not
only reduced cell death but also precocious surface migration in swarming development.

Previously, our laboratory had demonstrated that a pair of two-component system, RssA-RssB, is involved in coordination of swarming behavior [19, 20], and further suggested that signaling of RssA-RssB controls the duration of swarming lag period in *S. marcescens* [Tsai et al., unpublished data]. We were wondering that whether RssA-RssB signaling modulates the proportion of dead cells during swarming lag period. While inoculating the *rssBA* null mutant onto swarming plate, most of the vegetative cells were live following the early swarming development, a phenomenon contradicted to the wild-type parental strain but resembled to *ccdAB*$_{Sm}$ deletion mutant.

Therefore, we hypothesize that PCD might be activated via RssAB two-component signal transduction system and CcdAB$_{Sm}$ TA module in *S. marcescens*. The underlying mechanism of this regulatory pathway will be further investigated in the future.
# Chapter 2: Materials and Methods

## 2.1 Bacteria strains, plasmids, and primers

### Table 2-1. Bacteria strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serratia marcescens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH-1</td>
<td>Clinical isolate</td>
<td>[19]</td>
</tr>
<tr>
<td>ΔrssBA</td>
<td>rssBA knockout mutant, Gm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Tsai et al., 2008</td>
</tr>
<tr>
<td>ΔccdAB</td>
<td>ccdAB&lt;sub&gt;sm&lt;/sub&gt; knockout mutant, Sm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F-&lt;phil&gt;80d&lt;sub&gt;lac&lt;/sub&gt;ΔM15,Δ&lt;sub&gt;(lacZYA-argF)U169&lt;/sub&gt;,&lt;br&gt;deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DB3.1</td>
<td>F- gyrA462 endA1 glnV44 (sr1-recA) mcrB&lt;br&gt;mrr hsdS20(rB-, mB-) ara14 galK2 lacY1&lt;br&gt;proA2 rpsL20 xyl5 _leu mtl1</td>
<td>PTT</td>
</tr>
<tr>
<td>CC118</td>
<td>λ-pir lysogen [ (ara-leu) araD lacX74 galE&lt;br&gt;galK phoA20 thi-1 rpsE rpoB argE(Am)&lt;br&gt;recA1]; permissive host for suicide plasmids requiring Pir protein</td>
<td>[46]</td>
</tr>
<tr>
<td>S17-1</td>
<td>λ-pir lysogen [thi pro hsdR hsdM+ recA RP4&lt;br&gt;2-Tc::Mu-Km::Tn7 (Tpr Smr)]; permissive host able to transfer suicide plasmid pDM4 to recipient cells via conjugation</td>
<td>[46]</td>
</tr>
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Table 2-2. The plasmid used in this study.

<table>
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<tr>
<td>pGEM-T Easy</td>
<td>TA cloning vector, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pBAD24</td>
<td>araC coding region, M13 intergenic region,</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>P&lt;sub&gt;BAD&lt;/sub&gt; promoter, pBR322 origin, Shine-Dalgarno box, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pUT-Sm</td>
<td>Suicide plasmid contains mini-Tn5 (Sm&lt;sup&gt;r&lt;/sup&gt;);</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>requires Pir protein for replication</td>
<td></td>
</tr>
</tbody>
</table>

The primers used in this study:

<table>
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<th>No</th>
<th>Primer name</th>
<th>Oligodeoxyribonucleotide sequence (from 5’ to 3’</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>EcoccdBABF</td>
<td>5’- CGAATTCCGCTGAATTGGCATACCCCTGTTT -3’</td>
</tr>
<tr>
<td>2</td>
<td>XbaccdABR</td>
<td>5’- CTCTAGATGATCGAACAGCACTTCACAGCGTCA -3’</td>
</tr>
<tr>
<td>3</td>
<td>Sal5ccdKOF</td>
<td>5’- AAGCACCTGACCTATATCGAGTCG -3’</td>
</tr>
<tr>
<td>4</td>
<td>Eco5ccdKOR</td>
<td>5’- GCTTTGAACGTCTAGTAAGTAGGG -3’</td>
</tr>
<tr>
<td>5</td>
<td>EcoccdBFB</td>
<td>5’- ACTGAAGAAAAATGGTTTGTGCTTGTCT -3’</td>
</tr>
<tr>
<td>6</td>
<td>Xba3ccdKOR</td>
<td>5’- CCACTTCCGCATGTACGACTACAT -3’</td>
</tr>
</tbody>
</table>

2.2 Bacteria medium and growth conditions

Bacteria were routinely cultured with agitation in Luria-Bertani (LB) broth at 30°C with adequate antibiotics when necessary.

2.3 Swarming motility assay

Swarming assays were performed on LB medium solidified with 0.8% (wt/vol) Eikan agar. 1 μl 10<sup>10</sup> CFU/ml overnight bacterial LB broth culture was inoculated onto the centers of LB agar plates and incubating at 30°C.
2.4 Bacterial LIVE/DEAD Staining

Counts of Viability bacteria were obtained using the LIVE/DEAD (L/D) stain (Invitrogen) contains a mixture of two components: SYSTO®9, a green fluorescent nucleic acid stain; and propidium iodide, a red fluorescent nucleic acid stain. SYSTO 9 generally stains damaged and intact bacteria whilst propidium iodide penetrates only bacteria with damaged membrane, competing with SYSTO 9 when both dyes are present. Incubated with the right mixture of dyes, intact or 'live' bacterial cells will fluoresce green whilst damaged or death bacterial cells will fluoresce red. Swarming plate were stained (1.5μl SYTO 9 and 1.5μl PI in final quantities) for 15 min at room temperature in the dark, followed by imaging by fluorescence microscope.

2.5 Postsegregational killing assay

CH-1ΔccdABSm containing the pBAD and pBAD-ccdABSm vector were grown overnight at 30°C in LB supplemented with kanamycin. Overnight cultures were centrifuged at 8,500 rpm for 3 min at room temperature and resuspended in the same volume of LB broth. Cultures were then 1:100 diluted in LB broth and grown at 30°C. Cultures were diluted every 45 min to maintain an OD 600 of 0.1 to 0.3. Samples were removed at 0, 45, 90, 135,180 and 225min, diluted in LB broth, plated on LB plates, and incubated at 30°C for counting.

2.6 Reverse transcription-PCR (RT-PCR) assay

Total bacterial RNA was extracted using a Trizol kit (Invitrogen, U.S.A). The relative amounts of transcripts from the genes tested were evaluated by the reverse transcription-PCR (RT-PCR) assay. When addressing genes regulated by RssA-RssB, RNA was isolated from strains CH-1 and ΔrssBA grown in LB broth culture for 5 hr and then reverse transcribed into cDNA with SuperScript III First-Strand Synthesis System kit (Invitrogen, U.S.A) according to the manufacture’s protocol. The expression level of genes tested was verified by primers designed to amplify the
coding sequences of these genes. 16S rRNA was used as the internal control to confirm that equal amounts of total RNA were used in each reaction. When addressing expression level of genes during swarming, bacteria was harvested from the swarming plates and the RNA was extracted. The conditions used to reverse transcribe the RNA and PCR were the same as verifying gene expression level in the broth culture corresponding to individual genes.
Chapter 3: Preliminary Results

3.1 Cell death during swarming development in S. marcescens

\textit{Serratia marcescens} swarming is a complex multicellular behavior comprising at least two morphological and physiological distinct cell populations: non-motile vegetative cells and highly-motile swarmer cells [13]. Since these vegetative cells are nonmotile, we addressed the cell viability in these vegetative cells by LIVE/DEAD stain (Invitrogen). Surprisingly, the dead cells were dramatically increased in the population of vegetative cells from 1.5 hr to 2 hr and existed at 3 hr and 4 hr vegetative cell population following swarming development. Even though dead cells were found in swarmer cell population, the ratio of dead cells was much lesser in swarmer cell population in swarming development (Figure 3-1A and B).

\begin{itemize}
  \item[A.] 1.5 hr
  \item[B.] 2 hr
  \item[3 hr swarmer cell] 3 hr vegetative cell
  \item[4 hr swarmer cell] 4 hr vegetative cell
\end{itemize}

\textbf{Figure 3-1 Population-specific cell death during swarming in S. marcescens CH-1.}

(A) Cells were stained by the use of LIVE/DEAD stain (Invitrogen) after inoculating onto the swarming plates at different time intervals to demonstrate the cell viability in swarming development. The ratio of viable cells was shown (B).
3.2 Identification of chromosomal TA system, \textit{ccdAB}_{Sm}, in \textit{S. marcescens}

To identify the genes involved in cell death in \textit{S. marcescens} swarming development, we screened the chromosomal toxin genes which have been demonstrated to cause bacterial cell death. Genomic analysis for the CidA, SkfA-H and 11 TA systems using TBLASTN-Search on the \textit{S. marcescens} genomic database (Sanger Institute) revealed the existence of a CcdB homolog (CcdB{	extsubscript{Sm}}; SMA0821) with a CcdA homolog (CcdA{	extsubscript{Sm}}; SMA0820) upstream of ccdB{	extsubscript{Sm}} (Figure 3-2). The finding of ccdA{	extsubscript{Sm}} and ccdB{	extsubscript{Sm}} genes agreed with the hypothesis that the TA modules may play essential roles in bacterial cell growth during adaptation to environmental stresses by inducing a state of reversible bacteriostasis \cite{31}. It also raises intriguing questions as to whether expression of \textit{ccdAB}_{Sm} may be regulated during swarming development and associated with programmed cell death.

A.

![Sequence alignment of CcdB{	extsubscript{Sm}} homolog and location of the CcdB{	extsubscript{Sm}} gene on the chromosome](image)

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\textit{S. marcescens} post-segregational killing (PSK) is one of the characteristics of bacterial TA systems. (A) Protein alignment results using SMA0821 amino acids sequence as a query. CcdB{	extsubscript{Sm}} has higher identities with those CcdB of \textit{Photorabdus luminescens}(Pl), \textit{Yersinia enterocolitica}(Ye), \textit{E. coli} (O157H7), \textit{shigella dysenteriae}(Sd), and \textit{Vibrio cholerae}(Vc). Amino acid residues identical are shown by red shades, and conservative substitution by blue shades. (B) The genomic sequence was from \textit{S. marcescens} Db11(Sanger Institute). ccdA{	extsubscript{Sm}} (SMA0820) and ccdB{	extsubscript{Sm}} (SMA0821) were colored in red and the flanking genes were colored in blue.

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Post-segregational killing (PSK) is one of the characteristics of bacterial TA systems.
systems [25]. Since the antitoxins are metabolically unstable, the newborn plasmid-free cells are killed by the stable toxins inherited from mother cells. In this way, TA loci prevent the proliferation of plasmid-free cells in growing bacterial cultures [49]. Therefore, we tested whether the CcdAB<sub>Sm</sub> is also able to mediate PSK in <i>S. marcescens</i> as an authentic TA module. The plasmid containing <i>cddAB<sub>Sm</sub></i> under the control of P<sub>BAD</sub> promoter (pBAD-<i>cddAB<sub>Sm</sub></i>) was transformed into a <i>cddAB<sub>Sm</sub></i> deletion mutant of CH-1 (<i>ΔcddAB<sub>Sm</sub></i>) to avoid the inference (counteract) of chromosomal <i>cddAB<sub>Sm</sub></i> in PSK. As shown in Figure 3-3, after 45 min of culture supplemented with 0.1% arabinose but without antibiotic selection, the ability of <i>ΔcddAB<sub>Sm</sub></i> / pBAD-<i>cddAB<sub>Sm</sub></i> to form colonies strikingly decreased in comparison with that of the control strain <i>ΔcddAB<sub>Sm</sub></i> / pBAD, indicating that the loss of pBAD-<i>cddAB<sub>Sm</sub></i> mediates PSK. Briefly, the <i>cddAB<sub>Sm</sub></i> is a functional TA module for plasmid stabilization in <i>S. marcescens</i>.

![Graph showing PSK](image)

**Figure 3-3** <i>cddAB<sub>Sm</sub></i> mediates postsegregational killing (PSK) in <i>S. marcescens</i>. <i>ΔcddAB<sub>Sm</sub></i> carrying the pBAD24 vector (●) and its derivative containing <i>cddAB<sub>Sm</sub></i> (▲) were grown as described in Materials and Methods, respectively. Exponential cultures in LB at 30°C were sampled at the times indicated in the figure. Surviving bacteria were scored as the number of CFU on LB plates at 30°C. Values corresponded to the means of results for three independent experiments.

### 3.3 <i>cddAB<sub>Sm</sub></i> is involved in cell death during swarming development

To elucidate the role of CcdAB in the life cycle of <i>S. marcescens</i> during
swarming, swarming behavior of $\Delta ccdAB_{Sm}$ was addressed. When being spotted onto swarming plates, cell viability was profoundly affected in the absence of $ccdAB_{Sm}$ compared to wild type cells (Figure 3-1 and 3-4). While the CH-1 cells viability was dramatically decreased in the population of vegetative cells after 1.5 hr of inoculation (Figure 3-1), deletion of $ccdAB_{Sm}$ reduced cell death from 1.5 to 4 hr during swarming development (Figure 3-4), indicated that $ccdAB_{Sm}$ is involved in cell death in swarming development.

Figure 3-4 Deletion of chromosomal $ccdAB_{Sm}$ reduces cell death during swarming. (A) LIVE/DEAD staining of $\Delta ccdAB_{Sm}$ strain in swarming development was shown after inoculating onto the swarming plates at different time intervals. (B) The ratio of viable cells of $\Delta ccdAB_{Sm}$ from LIVE/DEAD stain was shown.

While $\Delta ccdAB_{Sm}$ performed swarming motility even though the cell death was reduced in swarming development, we investigated the swarming behavior of $\Delta ccdAB_{Sm}$ in more details. Interestingly, a precocious swarming phenotype was observed in $\Delta ccdAB_{Sm}$ strain (Figure 3-5A). Further characterization of this mutant indicated that the duration of lag period but not the migration velocity of $\Delta ccdAB_{Sm}$
was affected in swarming development (Figure 3-5B). Coupled with the results of LIVE/DEAD stain and precocious swarming behavior of ΔccdABSm, we supposed that the TA system, CcdABSm, involved in cell death and delay the initiation of surface migration in swarming development in *S. marcescens*.

**A.**

**Figure 3-5 The effect of ccdAB TA system during swarming development.** (A) Swarming motility assay of *S. marcescens* strains CH-1, isogenic mutant ΔrssBA and ΔccdAB were performed. (B) Bacterial swarming diameter of CH-1(black), ΔrssBA(red) and ΔccdAB(green) were shown. Overnight bacterial LB broth culture (1 μL, 10^10 CFU/ml) was inoculated onto 0.8% (wt/vol) Eiken agar LB plates at 30°C for swarming motility assay.

**B.**

3.4 RssAB affects cell death in swarming development

RssAB is a pair of two-component system regulating swarming initiation in *S.
marcescens (Tsai YH et al., unpublished data and Figure 3-5). Since the deletion of ccdABSm shortened the duration of lag period in swarming development (Figure 3-5), which was resemble the precocious swarming phenotype of ΔrssBA. We questioned whether deletion of rssBA reduces cell death in swarming development as ΔccdABSm. Consistent with our expectation, most of the ΔrssBA cells including swamer cells and vegetative cells were live during swarming development from 1.5 to 4 hr (Figure 3-6 A and B), a phenomenon completely differed from the wild type CH-1 but resemble ΔccdABSm strain (Figure 3-1, 3-4 and 3-6).

**Figure 3-6 RssAB regulates cell death in swarming development.** (A) ΔrssBA cells were stained by LIVE/DEAD stain (Invitrogen) after inoculating onto the swarming plates at different time intervals. (B) The ratio of viable cells of ΔrssBA was shown. (C) Bacterial growth curves of CH-1(black), ΔrssBA(red) and ΔccdAB(green) in swarming development were shown. Each point on the curve is an average from triplicate experiments. Overnight bacterial LB broth culture (1 μL, 10^{10} CFU/ml) was inoculated onto 0.8% (wt/vol) Eiken agar LB plates at 30°C for swarming assay.
Furthermore, growth curve of these strains in swarming development demonstrated that the growth of wild type cells was arrested at 2 hr after inoculation onto the swarming plates (Figure 3-6C). However, ΔccdB_{Sm} and ΔrssBA strains grown normally without obvious arrest in swarming development (Figure 3-6C). Taken together, these results suggested that RssAB involves in regulation of cell death in swarming development.

3.5 RssAB down-regulates expression of ccdAB_{Sm}

We have demonstrated that both ΔrssBA and ΔccdB_{Sm} strains had much lesser dead cells through swarmer and vegetative cell population compared to wild type CH-1 during swarming development (Figure 3-1, 3-4 and 3-6). Whether RssAB signaling involves in the regulation of CcdAB_{Sm} system to mediate cell death in S. marcescens was addressed. Since RssA-RssB signaling was turned on in swarming lag phase (2 hr) and turned off once swarming had begun (3 hr) and also turned on at 5 hr in LB broth culture [Tsai et al., unpublished data], examined the expression of ccdAB_{Sm} at these different growth conditions were examined.

![Figure 3-6](image)

**Figure 3-6** Expression of ccdAB_{Sm} was regulated by RssAB in swarming development and LB broth culture. (A) The expression level of ccdAB_{Sm} was down-regulated by RssAB in S. marcescens CH-1 cells at 2 hr in swarming lag phase compared to 3 hr when surface migration was initiated and showed differential expression in CH-1 and ΔrssBA at 5 hr in LB broth culture. (B) Total RNA was extracted from CH-1 or ΔrssBA strain at 3 hr, 4 hr, 5 hr and 6 hr in LB broth culture. The expression level of ccdAB_{Sm} was assessed by RT-PCR.

As shown in Figure 3-6A, the transcription of ccdAB_{Sm} was elevated in ΔrssBA.
On the other hand, only 5 hr of \( ccdAB_{Sm} \) expression showed differential expression level between CH-1 and \( \Delta rssBA \) strains in LB broth culture (Figure 3-6B). These results indicated that RssAB signaling down-regulates \( ccdAB_{Sm} \) expression in \( S. marcescens \) and suggested that RssA-RssB signaling may temporally control \( S. marcescens \) swarming development through regulating \( ccdAB_{Sm} \) expression to activate \( CcdB_{Sm} \) leading to cell death.

**Figure 3-7** \( ccdAB_{Sm} \) co-transcribe with upstream genes but not downstream genes. (A) The physical map flanking \( ccdAB_{Sm} \) on Db11 chromosome was shown. The location of primers used for amplification were shown. The A primer pair (red) were used to amplify \( ccdAB_{Sm} \) with upstream gene and the B primer pair (green) were used to amplify \( ccdAB_{Sm} \) with downstream gene. Primers used to amplify \( ccdAB_{Sm} \) in Figure 3-6 were indicated in yellow. (B) Total RNA was extracted from CH-1 cells or \( \Delta rssBA \) cells at 5 hr LB broth culture. Both A and B primer pairs could get PCR products when using \( S. marcescens \) CH-1 genomic DNA as a template (data not shown). While using cDNA as a template, we could get PCR product by A but not B primer pairs.

To further characterize how RssAB down-regualtes expression of \( ccdAB_{Sm} \),
electrophoretic mobility shift assay (EMSA) was performed to assess the binding capacity of RssB–P to $ccdAB_{Sm}$ promoter. However, the result from EMSA demonstrated that 400 bp DNA fragment upstream $ccdAB_{Sm}$ was not directly bound by RssB–P (data not shown). Since the distance between $ccdAB_{Sm}$ and their upstream genes is 82 bp (Figure 3-7A), we thus wondered whether $ccdAB_{Sm}$ co-transcribed with upstream genes and RssAB signaling regulates the transcription of these genes instead of $ccdAB_{Sm}$ only.

RNA extraction from *S. marcescens* CH-1 cells was employed in RT-PCR to determine whether $ccdAB_{Sm}$ and sma0819 form an operon and whether transcription is coupled to surrounding open reading frames. As shown in Figure 3-7, the combination of primers spanning sma0819 and $ccdAB_{Sm}$ resulted in a PCR product of the expected size but the combination of primers spanning $ccdAB_{Sm}$ and sma0822 did not. Furthermore, compared with the RNA extracted from ΔrssBA cells at 5 hr LB broth culture, the transcript of sma0819- $ccdAB_{Sm}$ was down-regulated in wild type cells. These results suggested that $ccdAB_{Sm}$ and sma0819 are transcribed from a polycistronic message and this transcript is regulated by RssAB signaling.
Chapter 4: Discussion and Future Work

In this study, we are attempting to characterize the phenomenon of cell death and the mechanism of how does it be regulated in the development of swarming behavior. Our preliminary results showed that both ΔrssBA and ΔccdAB_Sm strains had much lesser dead cells through swarmer and vegetative cell population compared to wild type CH-1 during swarming development (Figure 3-1, 3-4 and 3-6). Moreover, deletion of ccdAB_Sm shortened the duration of lag period in swarming development (Figure 3-5), which was resemble the precocious swarming phenotype of ΔrssBA strain [Tsai et al., unpublished data]. Furthermore, we provided the evidence that RssAB down-regulates ccdAB_Sm transcription at the time when RssAB signaling is ON. These results suggested that RssAB signaling might activate CdB_Sm to induce cell death in S. marcescens in early swarming development.

To answer how does RssAB signaling regulate expression of ccdAB_Sm, EMSA was conducted to address the interaction between RssB~P and hypothetical promoter of ccdAB_Sm (the 400 bp DNA fragment upstream of ccdAB_Sm). Unexpectedly, this DNA fragment was not directly bound by RssB~P (data not shown). We thus doubted whether ccdAB_Sm co-transcribed with upstream genes and this transcript is regulated by RssAB signaling. As shown in Figure 3-7, ccdAB_Sm indeed co-transcribed with upstream gene sma0819 and this transcript was also regulated by RssAB. Protein BLAST of ccdAB_Sm upstream genes (sma0815 ~ sma0819) suggested that gene products of sma0816 ~ sma0819 are components of putative oligopeptide ABC transport system and sma0815 encodes a putative L-asparaginase. To address whether RssAB down-regualtes expression of this operon including ccdAB_Sm, EMSA would be performed to assess the binding capacity of RssB~P to promoter of sma0815. In addition, it has been suggested that the activation of TA toxin such as MazF may dependent on extracellular death factor (EDF), a signaling oligopeptide which could
be processed by Asparagine synthetase A, to cause cell death [50]. It is very interesting to characterize the upstream genes of $\textit{ccdAB}_{\text{Sm}}$ and further examine their relationship with $\textit{ccdAB}_{\text{Sm}}$ and cell death.

Swarming is a complex multicellular behavior, which required various signals in regulatory pathways that lead to swarming behavior of different model bacteria. It has now become clear that many of these pathways also affect the formation of biofilms, surface attached bacterial colonies[51]. We have demonstrated that $\textit{ccdAB}_{\text{Sm}}$ was involved in cell death in swarming development and this phenomenon was regulated by RssAB. The fact that RssAB influence both swarming development and biofilm maturation suggested that RssAB may influence cell death in both surface associated multicellular behavior [Tsai, Y.H. et al., unpublished data]. Moreover, it is recently reported that chromosomal TA systems mediate cell death in $\textit{E. coli}$ biofilm formation[52]. We would further address whether CcdAB$_{\text{Sm}}$ is also involved in cell death in biofilm formation in $\textit{S. marcescens}$. Taken together, we would like to reveal the mechanism of how bacteria coordinate cell death when living on a surface.
Chapter 5: References


